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Growth Promotion in Elephant Foot Yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) Consequent to Colonization by the Root Endophytic Fungus, *Piriformospora indica*

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Abstract

Elephant foot yam (EFY) is widely cultivated in many states of India due to its high production potential; acceptability as a vegetable in many delicious recipes and use in Ayurvedic medicines. *Piriformospora indica* is an endophytic mycorrhiza like fungus which has shown pronounced growth promotional and disease suppressing activities in many crops. The potential of *P. indica* to colonize elephant foot yam roots was explored for the first time and growth promotion in elephant foot yam plants consequent to root colonization by *P. indica* was also studied. Among twelve solid media evaluated for growth, maximum radial growth of *P. indica* was obtained on cassava starch and wheat extract + Jaggery (9 mm day^{-1}). Among the ten liquid media tested, maximum mycelial mass was observed in wheat extract + jaggery and wheat extract + PDA media. The colonization ability of *P. indica* in elephant foot yam (var. Gajendra) was studied by trypan blue staining and further confirmation of colonization was done by amplifying species specific *Pitef1* gene. Incorporation of *P. indica* resulted in promotion of all growth parameters like shoot length, root length, biomass, girth, leaf area etc. of host plant. *P. indica* is known to inhibit many pathogens in various crops apart from causing growth promotion. Hence, studies on the effect of *P. indica* colonization on various diseases of elephant foot yam will help in tapping the full potential of the organism in organic cultivation of EFY.

Keywords: Elephant foot yam, *P. indica*, colonization, media, growth promotion

Introduction

Amorphophallus paeoniifolius (Dennst.) Nicolson, commonly known as Elephant foot yam (EFY) belonging to the Araceae family is one of the most popular and profitable underground stem tuber crop which has nutritional as well as medicinal value. The crop offers remarkable scope as cash crop and has much popularity due to its shade tolerance, easiness to cultivation, high productivity, steady demand and good price (Ravi et al*.,* 2011). Current crop production systems target the exploitation of sustainable techniques by maintaining a balance with the environment. A large number of beneficial microbes have been located and have showed remarkable results (Lakshmipriya et al., 2016). The root endophytic fungus, *Piriformospora indica*, is a microbe with the potential to offer plentiful benefits and opportunities in modern crop production system (Mensah et al., 2020). This root endophytic fungus of the order Sebacinalesis capable of colonizing roots and forming symbiotic relationship with plants. The fungus offers benefits to plants by improving plant growth, imparting stress resistance, and producing secondary metabolite or phytochemical production (Varma et al*.,* 1998; Tsimilli-Michael and Strasser, 2013; Dolatabadi et al*.,* 2017).The organism could be easily cultivated on several synthetic media and has typical pear shaped chlamydospores (Varma et al., 1998; 1999; 2001). The morphology of the mycelium and the growth rate varies depending on the composition of the nutrients of the culture medium. Maximum spore yield and dry cell weight

were obtained in modified Hill–Kaefer synthetic medium under the optimized culture condition (Hill and Kafer, 2001; Pham et al., 2004). Coconut water, a waste product from the coconut industry was used as a medium for the cultivation of *P. indica* (Anith et al., 2015). *P. indica* helps in growth promotion by increasing nutrient uptake, allowing plants to survive under drought, high- and low-temperature and salt stresses, eliciting systemic resistance to toxins, heavy metal ions and pathogenic organisms, and thus stimulates growth and seed production. Colonization by *P. indica* resulted in growth promotion, corm yield increase and delayed disease incidence in taro varieties viz., Sree Kiran and Muktakeshi, susceptible and resistant to *Phytophthora colocasiae,* respectively (Lakshmipriya et al*.,* 2016). This paper reports the successful root colonization in *Amorphophallus paeoniifolius* by *P. indica* for the first time, which resulted in significant enhancement of plant biomass.

Materials and Methods

Piriformospora indica culture for the present study was obtained from Amity Institute of Microbial Sciences, Amity University, India. The culture was maintained on PDA slants/ plates at room temperature (28 ± 2ºC). Corms of elephant foot yam variety, Gajendra were obtained from ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram.

Evaluation of media for the multiplication of *P. indica*

Solid media: Twelve solid media were evaluated for the cultivation of *P. indica.* The media used for the study were Potato Dextrose Agar (PDA); PDA + Jaggery 2%; PDA + root extract of *Carex hirta,* Cyperaceae 1%; PDA + root pieces (the roots of *C.hirta*) 1%; Jaggery (4%); Cassava starch agar (extract of tubers of cassava variety Sree Pavithra) 20%; tender coconut agar; rice water agar; malt extract agar (MEA); wheat extract agar (WEA); 50% wheat extract + 50% PDA and 50% wheat extract + 50% Jaggery. These media were autoclaved and dispensed into sterile Petri dishes. Mycelial discs of actively growing culture of *P. indica* was inoculated into the center of the Petri plates and incubated at 28 ± 2ºC and recorded the growth of mycelia at an interval of 24 hrs until 7 days of inoculation.

Liquid media: Ten media (natural and synthetic) were used to compare the mycelia mass production by the fungus. The media used were potato dextrose broth; tender coconut broth; cassava starch broth; jaggery broth (4%); rice water broth; malt extract broth; PDB + jaggery; wheat extract + PDB; wheat extract + jaggery and wheat extract broth. The mycelia discs of *P. indica* were inoculated into the Erlenmeyer flasks containing 100 ml of the above media. The inoculated flasks were incubated for 13 days at 28 ± 2ºC at 120 rpm in an orbital shaker and observed for mycelial growth. After incubation, mycelial clumps were harvested by filtering the contents through cheese cloth. The mycelial clumps were weighed and compared the mass.

The data were statistically analyzed using SAS statistical software (SAS 2010 – SAS Institute Inc., Cary, North Carolina, USA).

Colonization study

Potting mixture was prepared in 1:1:1 ratio (sand: soil: FYM). PDA broth was used for the preparation of *P. indica* inoculum. The mycelial clumps were mixed with sterile sand at 1% w/v. The plastic pots were filled with *P. indica* amended potting mixture. EFY side cormels approximately weighing 75 g were washed thoroughly with sterile distilled water for three times and then dipped in 4% jaggery solution to facilitate the adherence of mycelium amended sand to the cormels. The treated cormels were planted in the pots. The pots were maintained in the net house conditions and growth was observed daily. One month after planting, the plants were uprooted and the roots were carefully excised and examined under microscope.

Detection of *P. indica* colonization in EFY roots by staining with trypan blue in lacto phenol

Staining was carried out as per the protocol described by Philip and Hayman (1970). The roots were excised using sterile blade and rinsed with sterile distilled water, boiled with 10% KOH for 5-10 minutes for softening the root tissues which was followed by neutralization with 2% HCl for 15-20 minutes. These treated roots were cut into 1cm long pieces, stained with 0.5% trypan blue (Lobachemi) in lacto phenol for 10 minutes and washed in lacto phenol solution for 15 minutes to remove the excess stain. Slides were prepared from these samples and then DPX mounted. The colonization was critically observed under microscope (Nikon Eclipse E200, Nikon Corporation, Japan).

Plant genomic DNA isolation and PCR analysis

Fresh roots were taken from elephant foot yam plants, which were colonized by *P. indica* (after 30 days of co-cultivation). The roots were washed thoroughly with sterile distilled water to remove soil contaminants and dirt. The total genomic DNA was isolated from roots by CTAB method (Lodhi et al., 1994). 100mg of young fresh roots were crushed into fine powder with liquid nitrogen and 1ml of extraction buffer (pre-warmed at 60ºC) was added to the tissue powder and centrifuged at 10,000 rpm for 1 minute. The supernatant was collected in a fresh tube which was followed by the addition of 5 µl proteinase K. The tubes were incubated at 65ºC for 30 minutes for cell lysis. After incubation, the samples were centrifuged at 10,000 rpm for 10 minutes at 27ºC, supernatant was collected, added10 µl of RNase and incubated at 37ºC for 1 hour. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and mixed well followed by centrifugation at 15,000 rpm for 10 minutes at 4ºC. Upper aqueous layer was transferred into fresh tube and added 0.8 volume of ice-cold isopropanol and then incubated at -20ºC for at least 1 hour or overnight. Then it was centrifuged at 15,000 rpm (12,000×g) for 10 minutes at 4ºC. The supernatant was discarded and the pellet was washed with 0.5ml of 70% ethanol by centrifugation at 15,000 rpm (12,000×g) for 5 minutes at 4ºC. This step was repeated twice. DNA pellet obtained was air dried at 37ºC for 30 minutes and dissolved in 30-50ìl sterile 1X TE buffer and stored at -21ºC for PCR analysis.

DNA of *P. indica* was amplified using species specific primer as previously described (Satheesan et al*.,* 2012). Primer sequence and reaction mix optimized is listed below.

PiTEF forward primer: 5'TCGTCGCTGTCAACAAGATG3'

PiTEF reverse primer: 5'GAGGGCTCGAGCATGTTGT3'

The components were mixed as follows. Sterile water- 19 ìL, Taq buffer- 2.5 µL, dNTP mix- 0.5 µL, Taq polymerase- 0.5 µL, forward primer- 0.5 µL, reverse primer- 0.5 µL, DNA-1.5 µL and mixed by gentle vortexing (Labnet vortex mixer, USA) and PCR amplification was performed in an Agilent Sure Cycler 8800 (Agilent Technologies, USA). The PCR regime consisted of 2 minute at 94°C, 35 cycles of 30 sec at 94°C, 1 minute at 55°C and 1 minute 30 sec at 72°C and finally 8 minute at 72°C. Amplified products were resolved on a 1.5% agarose (Himedia) gel containing 0.5 µg ml^{-1} (0.5 µL) ethidium bromide and photograph was scanned through the Gel Doc System (Alpha Imager, Alpha Innotech, USA). The amplification products were stored at -20°C. The amplified products were purified to remove excess primers and nucleotides using a Nucleospin® gel and PCR cleanup kit (Macherey Nagel). The purified product was sequenced with the same primers as for the PCR amplifications. The sequencing was performed using Applied Biosystems® 3500 Genetic Analyzer, Life Technologies at Agri-genome, Cochin. The nucleotide sequence was determined by nucleotide BLAST (Basic Local Alignment Search Tool) search programme of NCBI. The gene sequences were also submitted to NCBI BankIt and accession numbers were assigned.

Plant growth promotion parameters

Plants colonized with *P. indica* as well as control plants were observed (9 plants) at 10 days intervals up to 45 days to determine the growth parameters including shoot length, root length, shoot weight, root weight, biomass etc. Since the plants were kept only for 45 days, no additional fertilizers were supplied to the plants. For the determination of the dry weight, the materials were dried in an oven at 40°C for 48 hrs.

Results and Discussion

Evaluation of media for the growth of *P. indica*

Solid media: The fungus, *P. indica* successfully grew in all the twelve media evaluated (Table 1). Unlike AM fungi, *P. indica* can be easily cultivated on a variety of synthetic media. Maximum growth was seen in cassava starch agar and the ability of the medium to support constant growth was clear from the growth recorded 4 days after inoculation (DAI) to 10 DAI (Fig. 1). This was followed by the medium, wheat extract + jaggery. The suitability of cassava, potato, sweet potato and taro as nutrient source in fungal media to grow *Aspergillus flavus*, *Bipolaris oryzae*, *Fusarium semitectum* and *Penicillium sp* had been reported earlier (Wongjiratthiti and Yottakot, 2017). Axenic propagation of *P. indica* in cultures makes their use practically feasible in the horticultural sector. This fungus is able to grow on axenic cultures of different media, where the best metabolizable carbon source was soluble starch and glucose (Serfling et al., 2007; Varma et al., 2014). Yadav et al. (2010) evaluated various

Table 1. Mycelial growth of *P.indica* in various solid media

Sl.No	Name of the medium	Mycelial growth in mm						
		4 (DAI)*	5 (DAI)	6 (DAI)	7 (DAI)	8(DAI)	9 (DAI)	10 (DAI)
1	PDA	25.33^{CD}	31.33^{C}	33.67^{D}	36.00^{E}	40.33^{E}	44.33^{E}	49.67^{F}
2	PDA + Jaggery	23.00^{DE}	27.33^{DE}	30.67^{E}	35.00^{EF}	40.33^{E}	45.33^{E}	50.33^{F}
3	PDA with root extract	21.00^{E}	24.33^{E}	27.33^{F}	33.00^{F}	34.00^{F}	38.67^{F}	42.33^{G}
4	PDA with root pieces	25.33^{CD}	27.00^{DE}	32.00^{DE}	35.67^{EF}	40.33^{E}	44.00^{E}	48.67^{F}
5	Jaggery (4%)	26.33^{BC}	29.33^{CD}	33.67^{D}	37.00^{E}	41.33^{E}	45.67^{E}	49.00^{F}
6	Cassava Starch agar	31.33^{A}	41.33^{A}	50.33^{A}	58.33^{A}	70.67^{A}	81.00^{A}	90.00^{A}
7	Tender coconut agar	10.67^{F}	13.67^{F}	14.67^{G}	16.67^{G}	17.67^{G}	19.00^{G}	20.67^{H}
8	Rice water agar	28.67^{AB}	35.00^{B}	39.33^{C}	46.00^{C}	55.67^{C}	63.00^{C}	70.00^{D}
9	Malt extract agar	28.67^{AB}	38.00^{AB}	45.33^{B}	55.67^{AB}	61.00^{B}	68.67^{B}	76.33^{C}
10	Wheat extract agar	27.67^{BC}	35.00^{B}	39.67^{C}	47.00^{C}	54.00^{C}	60.67^{C}	75.67^{C}
11	Wheat extract + PDA	20.67^{E}	26.00^{DE}	33.67^{D}	41.00^{D}	49.67^{D}	54.67^{D}	60.67^{E}
12	Wheat extract + Jaggery	28.67^{AB}	36.67^{B}	45.67^{B}	54.33^{B}	64.33^{B}	71.67^{B}	80.00^{B}
CD at 1%	2.8433	3.5306	2.9816	2.704	3.5939	3.1343	3.391	

*DAI- Days after inoculation

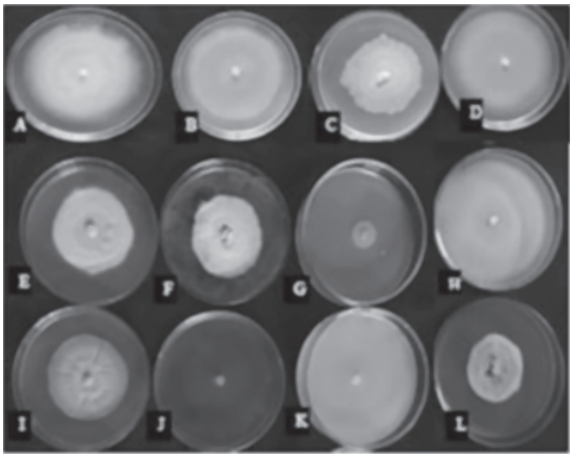

Fig. 1. Mycelial growth of *P. indica* on different solid media
A. Malt extract agar B. Wheat extract agar
C. Wheat extract + PDA D. Wheat extract+ Jaggery
E. Potato dextrose agar (PDA) F. PDA with root pieces
G. Tender coconut agar H. Cassava starch agar
I. PDA + Jaggery J. 4% Jaggery
K. Rice water agar L. PDA with root extract

nutrient media for culturing *P. indica* and reported suitability of jaggery based medium for culturing the fungus. Least growth was noted with tender coconut agar followed by PDA amended with root pieces. However, Anith et al. (2015) reported that coconut water-based medium was most appropriate for the mycelia production.

Liquid media: With continuous shaking in an orbital shaker at 120 rpm yielded mycelial aggregates resembling globose balls. No basidial formation was noted in the culture. After 13 days of incubation, the mycelial aggregates were harvested and fresh weight was recorded. *P. indica* could be cultivated on different media without a host, easily producing large quantities of fungal biomass (Varma et al., 1998). Among the media, maximum mycelial mass weight was obtained from the wheat extract + Jaggery broth and it was on par with wheat extract + PDB and PDB +Jaggery (Table 2). Tender coconut broth and cassava starch broth showed minimum mycelial mass weight (Fig. 2). Even though, cassava starch agar showed maximum linear growth of *P. indica*, the yield of mycelia was less.

Table 2. Mycelial growth of *P.indica* in different liquid media

Treatment	Medium	Mycelial weight(wet) in g
1	PDB	23.64^{C}
2	PDB + Jaggery	29.91^{AB}
3	4% Jaggery	26.09^{BC}
4	Cassava starch	16.48^{D}
5	Tender coconut	6.33^{E}
6	Rice water	22.85^{C}
7	Malt extract	25.70^{BC}
8	Wheat extract	22.55^{C}
9	Wheat extract + PDB	34.00^{A}
10	Wheat extract + Jaggery	34.13^{A}
CD (1%)	= 4.9324	

Means with at least one letter common are not statistically significant

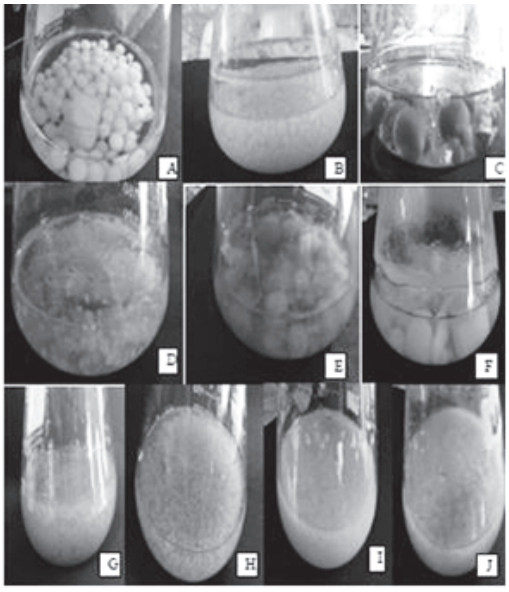

Fig. 2. Mycelial growth of *P. indica* in different broth

A. PDB
B. PDB+Jaggery
C. 4% Jaggery
D. Cassava starch
E. Tender coconut
F. Rice water
G. Malt extract
H. Wheat extract
I. Wheat extract +PDB
J. Wheat extract +Jaggery

Colonization of *P. indica* in roots of *A. paeoniifolius*

Staining with Trypan blue indicated the presence of chlamydospores inside the root cells of host plant (Fig. 3). Dark blue coloured chlamydospores were observed on the cortex of the root cells. Colonization of *P. indica* was further confirmed using *P. indica* - specific gene PiTEF1 primers.

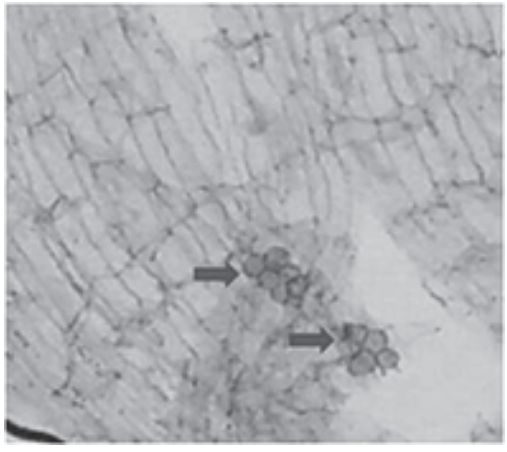

Fig. 3. Trypan blue stained chlamydospores of *P. indica* in the roots of *A. paeoniifolius*

PCR analysis using PiTEF1 primers produced an amplicon of 250 bp from the genomic DNA of the colonized roots which reconfirmed the successful colonization of the fungus in roots of elephant foot yam (Fig. 4). The sequence was submitted to NCBI BankIt with accession no. MH764588.1. This is the first report of successful colonization of *P. indica* in EFY. Lakshmipriya et al. (2016) reported successful colonization of *P. indica* in taro varieties viz., Sree Kiran and Muktakeshi. *P. indica* is widely distributed as a symptomless root endophyte, and it colonizes members of bryophytes, pteridophytes, gymnosperms and angiosperms (Varma et al., 2012).

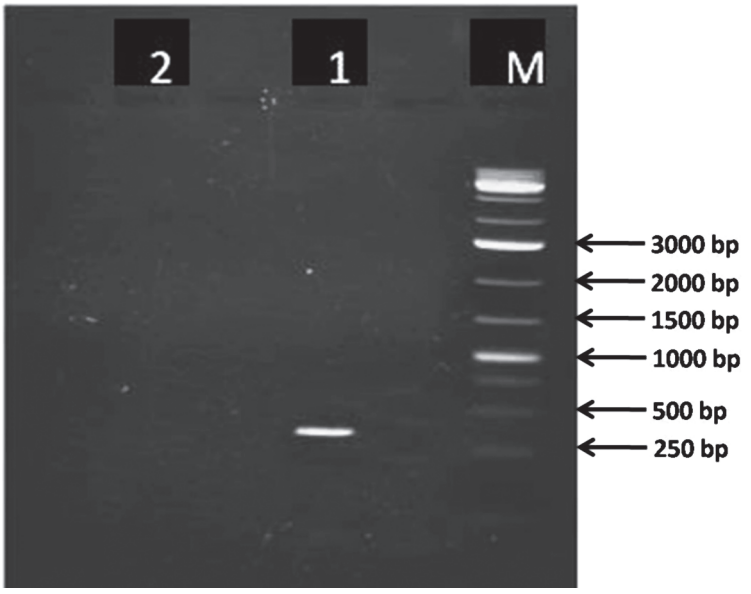

Fig. 4. Confirmation of *P. indica* by PCR using Pitef1 primers M: 1kb plus marker, Lane 1: Amplification from endophyte colonised EFY roots, Lane 2: Control

Growth promoting effect of *P. indica* colonization on host plant biomass

P. indica colonization resulted in a rapid increase in biomass of the host plant. The enhancement was visible from 10 days of co-culture to 45 days of culture (Fig. 5). There was also a significant increase in length and weight of root and shoot in colonized plants when compared with the control plants (Table 3). It was evident from most of the earlier reports that colonization of this endophytic fungus increased the plant growth and yield characteristics in many crops including black pepper (Anith et al., 2018); taro (Lakshmipriya et al., 2016); passion fruit (Yan et al., 2021); trifoliate orange (Yang et al., 2021); solanaceous plants (Vysakhi and Anith, 2021) and medicinal plants like *Adhatoda vasica,* where the endophytic fungus allows the plants to survive under drought, high- and low-temperature

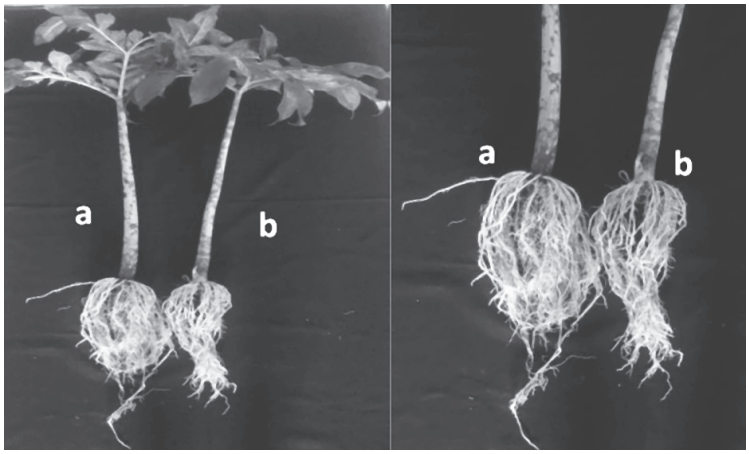

Fig. 5. Growth promotion effect of endophytic fungus in EFY
(a) *P. indica* colonized plant (b) Control plant

Table 3. The effect of *P.indica* colonization in plant growth

	Control	Treatment
Plant biomass(g)		
(a) Fresh biomass	132.3	239.9
(b) Dry biomass	44.1	79.9
Leaves number	73	130
Shoot		
(a) Shoot length(cm)	49	57
(b) Shoot weight(g)	70.1	81.0
(c) Shoot width(cm)		
(i) Top width	3.8	4.9
(ii) Middle width	5.0	7.0
(iii) Bottom width	7.1	8.2
Root length (cm)	29.7	41.5

and salt stresses (Rai and Varma, 2005). *P. indica* colonization significantly involves in growth stimulation and yield promotion in a vast range of plants (Varma et al., 1998; Fakhro et al., 2010). *P. indica* is a well-established symbiont for plant growth, development, and increased tolerance/resistance against invading pathogens in a vast host plant range. *P. indica* promotes biomass production by enhanced nutrition uptake and stimulates the development of plants, which may result in earlier flowering and faster fruit stetting (Yan et al., 2021).

The underlying growth promoting effect of this endophytic fungus has not been fully recognized, although several factors have been implicated in previous studies (Lakshmipriya et al*.,* 2016; Anith et al*.,* 2018; Vysakhi and Anith, 2021). The recent *P. indica* genome annotations will immensely support research and development programmes related to the fungus. The current exploitation of *P. indica* for its biotechnological applications in horticultural plants makes it important for future research to focus on deciphering its operational mechanisms. It is also necessary to evaluate the ecological and environmental implications, as well as economic benefits in horticultural industry.

This is the first report of successful colonization of *A. paeoniifolius* roots by *P. indica.* Considering the reports on induction of resistance and pathogens suppression by *P. indica*, the reaction of pathogens of EFY consequent to *P. indica* colonization may be studied in detail. Growth promotion and nutrient uptake coupled with pathogen suppression will help in tapping the full potential of *P. indica* in tuber crop production.

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